

From the Department of Histology, University of Lund
Lund, Sweden

**AUTONOMIC NERVOUS CONTROL
OF
OVARIAN FOLLICULAR CONTRACTILITY**

BY

BENGT WALLES

Lund 1977

From the Department of Physiology, University of Lund,
Lund, Sweden

AUTONOMIC NERVOUS CONTROL
OF
OVARIAN FOLLICULAR CONTRACTILITY

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten
i Lund för vinnande av medicine doktorsexamen kommer
att offentligen försvaras å Histologiska avdelningens före-
läsningssal (nya byggnaden), Institutionen för anatomi och
histologi, Lund, torsdagen den 2 juni 1977 kl. 9.15 (precis)

av

BENGT WALLES

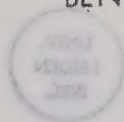
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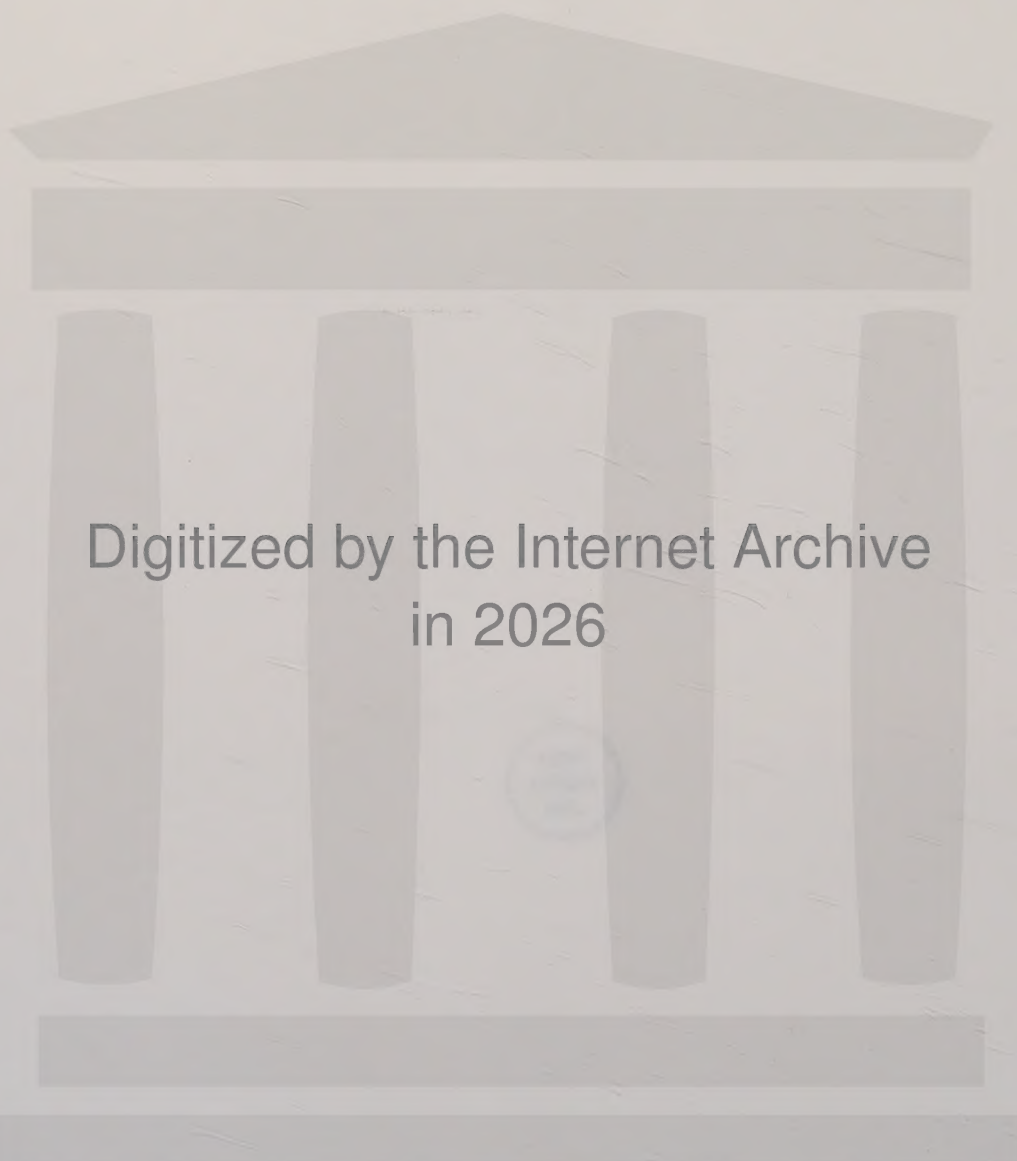
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Lund 1977

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The present dissertation is based on the following papers:

1. WALLES, B., L. EDVINSSON, G. NYBELL, CH. OWMAN and N.-O. SJÖBERG: Amine-induced influence on spontaneous ovarian contractility in the guinea-pig and the cat. Fertil. Steril. 25, 602-611, 1974.
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4. OWMAN, CH., N.-O. SJÖBERG, K.-G. SVENSSON and B. WALLES: Autonomic nerves mediating contractility in the human Graafian follicle. J. Reprod. Fert. 45, 553-556, 1975.
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I. EARLY INVESTIGATIONS ON THE PRESENCE OF CONTRACTILE ACTIVITY, SMOOTH MUSCULATURE, AND AUTONOMIC NERVES IN THE MAMMALIAN OVARY

The contractile properties of ovarian tissue have been known for more than one century (e.g. Rouget 1858). In 1921, Guttmacher and Guttmacher were able to demonstrate on porcine material that contraction of follicular wall preparations could be induced by administration of barium chloride, hydrochloric acid, and physostigmine, whereas relaxation was seen after adrenaline and atropine (subsequent to contraction with physostigmine). Ovarian contractile activity has since been studied under a variety of conditions both in whole ovaries, pieces of ovarian tissue or follicles (e.g. Lipner and Maxwell 1960 and Rocereto et al. 1969).

Although it has thus been known for a long time that ovarian tissue is able to contract, the structural basis for this property has been a matter of much dispute. Already in 1849 von Kölliker reported that non-vascular smooth muscle cells were a structural constituent of the ovary, and his observations were supported by Rouget (1858) and Aeby (1859). During the subsequent decades a number of studies followed which were based on histological examinations of ovarian tissue from a variety of mammals as well as other vertebrates (e.g. Grohe 1863, Winiwarter 1909, Guttmacher and Guttmacher 1921). Available microscopic techniques did not, however, provide any clear proof of the muscular nature of these so-called "smooth muscle cells". In an attempt to reinterpret the problem, Claesson (1947) applied a highly specific technique for visualization of the filamentous material in smooth muscle cells by polarization microscopy. The negative results of this investigation led Claesson (1947) to deny the existence of non-vascular contractile cells in the ovary, and after his report the interest in ovarian smooth musculature abated for many years, and it returned only recently when specific histochemical techniques and electron microscopy could be applied to the identification of smooth muscle elements in the ovary.

Early investigations (Frankenhäuser 1867, Waldeyer 1870, von Herff 1892, Winterhalter 1896) described that the intrinsic nerves of the ovary enter at the hilus along with the ovarian artery to supply the follicles as well as the ovarian vessels. Riese (1891), von Herff (1892), Winterhalter (1896) and others claimed that non-vascular nerves penetrated all the layers in the follicle, i.e. the theca externa, theca interna and the granulosa layer, while Abel and McIlroy (1912/13) found that only the two outer layers were reached by nerves. Also Retzius (1893), de Vos (1894) and Mandl (1895) found nerves in the ovary, though they held that the fibres did not penetrate the follicle at all except when accompanying blood vessels. Guttmacher and Guttmacher (1921) found, with a metylene blue staining technique, a rich plexus of nerve fibres in the theca externa of porcine Graafian follicles. These nerves were seen to run independent of the perivascular network and gave "a definite impression of bearing a functional relation to the muscle cells of the theca".

The methods used in these early studies on ovarian innervation were, however, of low specificity which made it almost impossible to separate different kinds of nerves. It was difficult also to show the fine branches of nerve endings and thus to make more precise studies of the intraovarian nervous system. It is thus obvious that detailed knowledge about the ovarian nervous and muscular systems and their possible functional relationship could not be achieved until a combination of various morphological and pharmacological techniques of high specificity was applied.

The aim of the present investigations has been:

- (1) to study the presence and distribution of (non-vascular) smooth muscle cells within the mammalian ovary;
- (2) to examine in detail the distribution of autonomic nerves (adrenergic and cholinergic) in the ovary with special regard to their localization in follicular structures;
- (3) to study the possible relationship of adrenergic and cholinergic nerve terminals with contractile cells in the follicular wall by ultrastructural techniques;

- (4) to elucidate whether nerve stimulation will evoke contractile activity in the follicular wall to secure the functional nature of the neuro-muscular complex; and
- (5) to characterize in detail the receptor mechanisms mediating the motor effects of transmitter amines on the ovarian smooth musculature.

II. ELECTRON MICROSCOPIC AND IMMUNOHISTOCHEMICAL EVIDENCE FOR THE PRESENCE OF (NON-VASCULAR) SMOOTH MUSCULATURE IN THE OVARY

Early histological staining techniques available have not provided sufficient specificity or selectivity for an unequivocal characterization and description of ovarian smooth muscle, and the polarization microscopic study by Claesson (1947) has often been cited as evidence for the absence of non-vascular smooth muscle cells in the mammalian ovary.

Electron microscopy

With the introduction of electron microscopy it became possible to identify elements within the ovary which, by their ultrastructural appearance, could be characterized as contractile cells. Such cells were first described by O'Shea (1970) and Osvaldo-Decima (1970) using rat and monkey material, respectively. Both authors found, in addition to fibroblasts in the follicular theca externa, also cells with many characteristics of smooth muscle cells. O'Shea (1970) found a third cell type which he considered as a transition form between fibroblasts and smooth muscle cells. The presence of smooth muscle-like cells in the ovary has been confirmed also for other mammalian species (see Papers 2, 6, and 7 for references).

In an electron microscopic study of the cow follicular wall (Paper 2), pieces from the protruding part of Graafian follicles were fixed in glutaraldehyde-osmium tetroxide. The theca externa was shown to contain groups of fusiform cells arranged essentially parallel to each other and to the surface of the follicular wall, and they were intermingled with fibroblasts and

bundles of collagen fibres. The cells contained myofilaments with a diameter of 6-8 nm, usually arranged in longitudinally running bundles, and were further characterized by dense bodies and a few micropinocytotic vesicles located at the inner surface of the cell membrane. Dense bodies were also found scattered in the cytoplasm, always in relation to the bundles of myofilaments. The cells thus closely resembled smooth muscle cells and were located as close as 30-100 nm from each other, sometimes with intermediary junction attachments. The cells had no obvious relation to the smooth musculature in the walls of blood vessels. In a few instances the muscle-like cells had certain ultrastructural features also found in fibroblasts: e.g. rough endoplasmic reticulum and a well-developed Golgi apparatus. Such cells may represent the transition form mentioned by O'Shea (1970).

Smooth muscle cells, defined on the basis of their ultrastructural appearance, are present in the theca externa also of human follicles, where they are by all probability responsible for the marked changes that can be induced in intrafollicular pressure (Paper 4).

Immunohistochemistry

The definite proof for the existence of contractile cells within the follicular theca externa has been obtained by immunohistochemical demonstration of the contractile proteins, actin and myosin, within the cytoplasm of theca cells in the rat follicle (Amsterdam et al. 1977; Paper 7). Incubation of ovarian cryostat sections with specific antibodies against smooth muscle myosin, followed by fluorescein-labelled anti- γ -globulin serum, resulted in an intense and well outlined fluorescence surrounding the entire circumference of the follicles. The fluorescent material was distributed in elongated cells forming several concentric layers within the theca externa. The layers of myosin-fluorescent cells were best developed in the wall of Graafian follicles and around corpora lutea, whereas thinner layers enclosed smaller follicles. Intense myosin fluorescence was also seen in the walls of the dense ovarian vascular plexus. Previous adsorption to smooth muscle myosin completely extinguished the fluorescence. Sections incubated with

anti-actin showed a similar fluorescence distribution. No fluorescence occurred in sections incubated with non-immune control globulin or in the presence of the fluorescein-labelled antiserum alone.

The theca externa of the ovarian follicle contains cells with the ultrastructural appearance of smooth muscle cells. Immunohistochemical demonstration of actin and myosin within the cytoplasm of theca externa cells proves the existence of contractile cells within this layer of the follicular wall.

III. HISTOCHEMICAL EVIDENCE FOR THE PRESENCE OF ADRENERGIC AND CHOLINERGIC NERVES IN THE OVARY

Adrenergic nerves

With the Falck-Hillarp fluorescence technique for visualization of catecholamines (see Björklund et al. 1972) it was convincingly shown in rabbits already ten years ago that the ovary is supplied with adrenergic nerve terminals having a close association with the follicles (Owman and Sjöberg 1966). This fundamental observation has been confirmed for several other mammalian species (for references, see Bahr et al. 1974) including man (Owman et al. 1967, Jacobowitz and Wallach 1967). In a study on bovine ovaries carried out with special regard to the follicular structures (Paper 2) it was shown that numerous adrenergic nerves were present in the ovarian stroma close to mature follicles. Several varicosed nerve terminals issued into the thecal layer to run isolated in an irregular manner; sometimes the fibres were seen to accompany small blood vessels. The arrangement of adrenergic nerve terminals was the same in the protruding part of the follicle, though they were less numerous than in the intraovarian portion of the follicular wall.

The human Graafian follicle was studied with the glyoxylic acid fluorescence method (Lindvall and Björklund 1974). Green-fluorescent adren-

ergic nerve terminals, running in an essentially meridional fashion, could be found in the wall of the whole Graafian follicle except for the most attenuated apex region. Away from the apex, the number of non-vascular nerve terminals increased, and it was highest in the intraovarian part of the follicle (Paper 4).

Chemical determination of the ovarian noradrenaline content has been carried out for several mammalian species (Table 1). These data confirm the fluorescence microscopic findings in general, and also that both the bovine and human ovary is well supplied with noradrenaline-containing (adrenergic) nerve terminals.

Cholinergic nerves

The possibilities to visualize cholinergic nerves selectively have their limitation in the fact that the commonly used method is indirect. The technique depends on the assumption that the presence of the enzyme acetylcholinesterase is a reliable marker of the cholinergic axon, provided pseudo-cholinesterase is inhibited by specific, irreversible antagonists, and the incubation time is maintained short enough to avoid reaction between any residual pseudo-cholinesterase and the substrate (Holmstedt 1957, Koelle 1963).

The cholinesterase technique was applied on ovarian tissue for the first time by Bulmer (1965) using rat, guinea-pig, rabbit, and monkey. When non-specific cholinesterase activity was inhibited, nerve fibres could be seen with an uneven distribution in the monkey ovary. The content of specific acetylcholinesterase was found to be very low in guinea-pig and rabbit ovaries, and in the rat ovary no specific reaction occurred at all. Jacobowitz and Wallach (1967) found cholinergic nerves in ovaries from cat, human and squirrel monkey. The fibres were distributed mainly around blood vessels though they were occasionally seen also in the stroma (cat and human) and within the interstitial tissue (monkey).

Specific acetylcholinesterase activity was demonstrated in the bovine ovary using Vibratome-sectioned tissue (Paper 5). A prominent system of cholinergic fibres was shown to run in the follicular wall. Some of them

TABLE 1

Ovarian content of noradrenaline

Mean values $\pm$ S.E.M. (number of determinations within parenthesis)

Species	Concentration ($\mu\text{g/g}$)		Reference
Human	0.86 ± 0.30	(5)	Owman <u>et al.</u> 1967
	0.57	(1)	Jacobowitz and Wallach 1967
Rhesus monkey	0.35 ± 0.06	(11)	Owman <u>et al.</u> 1977
Squirrel monkey	0.17	(1)	Jacobowitz and Wallach 1967
Cow	1.96 ± 0.19	(5)	Owman <u>et al.</u> 1977
Pig	0.25 ± 0.04	(5)	Owman <u>et al.</u> 1977
Cat	4.93 ± 1.30	(4)	Rosengren and Sjöberg 1967
	4.55 ± 1.08	(6)	Jacobowitz and Wallach 1967
Rabbit	0.20 ± 0.02	(11)	Sjöberg 1968
	0.223 ± 0.024	(16)	Bodkhe and Harper 1972
Guinea-pig	2.29 ± 0.35	(5)	Owman <u>et al.</u> 1977
left	2.32 ± 0.30	(4)	Kulkarni <u>et al.</u> 1976
right	2.16 ± 0.20	(4)	Kulkarni <u>et al.</u> 1976
Hamster	0.41 ± 0.09	(5)	Owman <u>et al.</u> 1977
Rat	0.50 ± 0.03	(6)	Owman <u>et al.</u> 1977
Mouse	0.63 ± 0.11	(6)	Owman <u>et al.</u> 1977
Hedgehog	0.86	(1)	Owman <u>et al.</u> 1977

enclosed blood vessels by a dense plexus. However, numerous nerves had no relation to the vascular bed, but formed a three-dimensional network in the thecal layer. The bulk of the fibres had a main direction essentially parallel to the follicular surface. The number of non-vascular nerves was largest in the intraovarian portion of the follicle as well as near the base of the protruding part. The axons then became fewer and were absent in the thin apex region.

With the same technique it was shown that also the human ovarian follicular wall is innervated by cholinergic nerves (Paper 4). These nerves have the same distribution in the follicle as the adrenergic ones, but are less numerous.

The Graafian follicular wall is innervated by adrenergic as well as cholinergic nerves as shown by specific histochemical techniques. Both types of nerves have the same distribution pattern: the density is greatest in the intraovarian portion of the follicle while the protruding part has a more sparse nerve supply. The number of adrenergic nerves exceeds that of the cholinergic fibres.

IV. STRUCTURAL EVIDENCE FOR A NEURO-MUSCULAR RELATIONSHIP IN THE FOLLICULAR THECA EXTERNA

Light microscopy

In attempts to correlate the distribution of sympathetic nerves with that of the ovarian smooth musculature (Paper 7), adjacent cryostat sections were reacted with glyoxylic acid to visualize the adrenergic transmitter (de la Torre and Surgeon 1976), and treated for immunohistochemical demonstration of actomyosin (Amsterdam et al. 1977). It was found that sympathetic axon terminals ran in between the contractile cells of the follicular theca externa. It was evident, though, that not all of the actomyosin-containing

cells were associated with fluorescent nerves. Comparison of consecutive sections also indicated that at least some of the non-vascular nerves in the ovarian stroma were related to the immunofluorescent cells scattered here. The more detailed relationship between the adrenergic nerves and the contractile cells was investigated at the ultrastructural level.

Electron microscopy

In an earlier study, O'Shea (1970) had found naked nerve axons in the rat ovary lying "closely related to smooth muscle cells". The actual distance between the nerve and muscle was, however, not reported in this study. In the cat and guinea-pig ovary, both myelinated (possibly sensory) and non-myelinated nerves have been described in the theca externa of mature follicles (Burden 1972). The non-myelinated nerves were located close to all cell types (fibroblasts, smooth muscle cells, and their transition forms) in the theca externa, and contained both dense-cored and clear vesicles. The varicosities were separated from the cells by a space of 200-300 nm. Since transmitter substance released from nerves within 20-1,000 nm of muscle cells may induce contraction (Burnstock 1970), the findings by Burden (1972) may indicate the presence of functioning synaptic contacts between the autonomic nerves and the smooth musculature within the follicular theca externa. The technique used by Burden (1972) (fixation of tissue in glutaraldehyde followed by osmium tetroxide) does not, however, allow for a separation between the two classes of postganglionic autonomic nerves (see Hökfelt and Nilsson 1965).

In a study on guinea-pig ovaries (Wallis et al. 1974), animals were injected with the false transmitter 5-hydroxydopamine, which is taken up and accumulated in the synaptic vesicles of adrenergic nerves (Tranzer and Thoenen 1967). The substance, which induces a high degree of electron density in the vesicles, can then serve as a marker of adrenergic nerves. On the basis of this method, the adrenergic nerve terminals could easily be distinguished, and were frequently found close to smooth muscle cells in the follicular theca externa. The membrane-to-membrane distance between the

nerve varicosity and the smooth muscle cell was in the order of 25-30 nm (Walles et al. 1974).

Electron microscopy of glutaraldehyde-osmium-fixed preparations from the protruding part of the bovine follicular wall showed small bundles of non-myelinated axons surrounded by Schwann cells in the theca externa (Paper 2). Some of these bundles could be traced to small blood vessels. Others came in close association with the groups of non-vascular smooth muscle cells. Here they lost part of their Schwann cell ensheathing, and showed axonal swellings. These varicosities contained small, 30-70 nm, vesicles which, in some of the axons, had a dense core. The axonal varicosities came as close as 50 nm from the membrane of the smooth muscle cells. This was interpreted as a true innervation contact, though membrane specializations were absent both at the axonal and muscular side of the adjacent membranes.

In the glutaraldehyde-osmium-fixed preparations it was possible to visualize one (smaller) population of axon varicosities containing electron-dense synaptic vesicles and another (larger) population in which the synaptic vesicles were only of the empty type. Adrenergic nerves are known to be characterized by dense-cored synaptic vesicles (Richardson 1964), but it is not always possible to demonstrate such vesicles if the preparations are fixed by glutaraldehyde and osmium (cf. Hökfelt and Nilsson 1965, Bloom and Aghajanian 1968). The findings thus indicated that adrenergic nerve terminals did exist in the theca externa, but it is possible that their number is greater than the number of axons in which electron-dense synaptic vesicles could be clearly visualized with the fixation technique used.

In order to obtain a better differentiation between adrenergic and non-adrenergic nerves, bovine follicle preparations were studied also after incubation in 5-hydroxydopamine followed by fixation in potassium permanganate (Paper 5). The two populations of axon varicosities (naked or partly enclosed by a Schwann cell ensheathing) could then be distinguished more unequivocally in the electron microscope: the majority contained vesicles of 30-60 nm with an electron-dense core representing the accumulation of

5-hydroxydopamine, which becomes visualized by the potassium permanganate fixation (Hökfelt 1968). Since this substance is selectively taken up into adrenergic nerves, the larger population of axons can be defined as adrenergic. The remainder of the varicosities had only empty vesicles of the 30-60 nm size. The absence of 5-hydroxydopamine accumulation in these indicates that they are non-adrenergic and, against the background of the results with the cholinesterase technique (Section III and Paper 5), it is assumed that they are of cholinergic nature. The varicosities of the cholinergic axons were seen to approach smooth muscle cells in a similar manner as those of the adrenergic nerves. The shortest neuro-muscular distance was usually about 150 nm.

Using principally the same methodological approach it was possible to demonstrate autonomic nerve terminals also within the theca externa of human Graafian follicles (Paper 4). Adrenergic terminals with dense-cored vesicles were seen to reach smooth muscle cells at a distance of about 100 nm. Occasionally, naked axon varicosities were found only 20 nm from the membrane of a muscle cell, further supporting the existence of functioning neuro-effector relations. A smaller number of nerve terminals was exclusively equipped with agranular 50 nm vesicles and these axons, too, were found in close apposition to smooth muscle cells. Such non-adrenergic nerves are probably identical with the acetylcholinesterase-containing fibres which were seen by light microscopy (Section III and Paper 4).

On the basis of histochemical and electron microscopic studies it is concluded that the adrenergic and cholinergic axons present within the follicular theca externa indeed innervate the system of smooth muscle cells in the sense that the membrane-to-membrane distance in the neuro-effector region is short enough to allow for effective diffusion of released transmitter onto the contractile elements.

V. NERVE-INDUCED CONTRACTION OF THE FOLLICULAR WALL: FURTHER EVIDENCE FOR A FUNCTIONING NEURO-MUSCULAR MECHANISM IN THE THECA EXTERNA

Release of the sympathetic transmitter can be evoked chemically by, for example, compounds (such as tyramine) that are actively taken up into the nerve terminals followed by displacement of the neuronal transmitter, or electrically by electrodes either directly applied to or in the nerves, or by transmural stimulation.

Experiments with tyramine

The contractile effect of noradrenaline released locally from the follicular nerves was analyzed in an in vitro system using bovine follicle preparations (Paper 2). Strips were dissected out from the protruding part of well-developed Graafian follicles. A silk ligature was tied around each end of the strip which was mounted longitudinally in an organ bath: one end was fixed near the bottom, the other connected to a force-displacement transducer. Isometric contractions were amplified and recorded on a polygraph. The bath contained a 37°C Krebs-Ringer solution continuously aerated with a mixture of 95 % O₂ and 5 % CO₂ to give a pH close to 7.40. Immediately after mounting, the ovarian strip was given a load of 1,000 dynes and was allowed to relax to a steady level of 500-600 dynes.

Tyramine is an indirectly acting sympathomimetic agent which releases noradrenaline from adrenergic nerves (Burn and Rand 1958, Langer et al. 1967). The specific effect of tyramine thus requires the presence of such nerves. Addition to the organ bath of increasing doses of tyramine produced a dose-dependent contraction of the follicle strip. The maximum response was, however, low: only about 50 dynes. After wash-out and after the strip tension had returned to the base-line, noradrenaline was administered to the bath, and the strip was thus exposed to the amine for 30 min in order to allow for an uptake into the adrenergic nerves known to be present in the preparation. About 5 min after noradrenaline had been washed out from the

bath, another sequence of tyramine doses was tested. The tyramine-induced contraction was now more pronounced, both in terms of a higher maximum contraction and a lower concentration for the half maximum response. After rinsing, the preparation relaxed to its original tension. Rinsing was then repeated several times in the course of 30-60 min. Following this, a cumulative dose-response test with tyramine was again performed. The new curve thus obtained was shifted towards higher tyramine concentration and its maximum was lower than in the previous experiment. If — after repeated sequences with tyramine tests — the strip was again exposed to noradrenaline, the high contractile response with tyramine could be restored. If cocaine (which inhibits the catecholamine uptake into adrenergic nerves; Hertting 1965) was added before the exposure to noradrenaline, the restoration of a high contractile response to tyramine was inhibited.

Electrical field stimulation

The lack of a separate nerve, accessible for stimulation, to the ovary makes it necessary to apply a technique for transmural stimulation (field stimulation) when the intraovarian autonomic nervous system is to be activated electrically.* Using principally the same set-up as for the studies of the tyramine effect on follicle strips, motor activity in the follicular wall was examined on human and bovine material (Papers 4 and 8, respectively). Stimulation was applied by two platinum electrodes placed one on either side of the strip preparation mounted in the organ bath.

Transmural activation by trains of biphasic pulses induced a frequency-dependent contractile response. The pulse duration was kept low (0.6-2.0 msec) with the aim of stimulating the nerves selectively without directly

* The first attempts to expose ovarian preparations to electrical stimulation were made by Okamura *et al.* (1975). However, no attempt was made in this study to stimulate the ovarian nerve system separately. The description of the stimulation parameters is too meagre to decide whether the nerves have, indeed, been activated. It is possible that the contractile responses obtained reflect direct electrical stimulation of the ovarian smooth musculature.

activating the smooth musculature in the strips. The selective activation of the nerves in the preparation was confirmed by administration of tetrodotoxin (Paper 5), which inactivates nerves through blockade of the Na^+/K^+ transport across the axonal membrane (Moore and Narakishi 1967). Under these conditions, the stimulation-induced response was completely abolished.

The innervation of the follicular smooth musculature by adrenergic as well as cholinergic nerves, and the fact that not only sympathomimetic compounds but also parasympathomimetics can contract the follicular wall, suggested that the follicular contraction induced by electrical stimulation might have involved both systems of nerves. This problem was elucidated in several types of experiments with electrical field stimulation (Papers 4 and 8) showing that: (a) blockade of the adrenergic nerves by the presence of guanethidine or bretylium markedly inhibited the contraction; (b) preincubation of the strip for 24 h at $+4^\circ\text{C}$ in reserpine, to deplete the adrenergic nerves of their transmitter, reduced the amount of contraction; (c) the presence in the bath of the alpha-receptor antagonists, piperoxan or phentolamine, almost abolished the contraction; and (d) the cholinergic receptor-blocking agents, atropine or hexamethonium, had little or no effect on the contractile response. It is concluded from these experiments that the contraction of the follicular wall, elicited by nerve stimulation under the present conditions, primarily involved the adrenergic type of axon terminals and associated alpha receptors in the smooth musculature.

Also a dilatory response can be obtained by electrical field stimulation, provided the alpha receptors are blocked and an active tonic contraction (by e.g. carbamylcholine) is induced in the preparation beforehand (Paper 8). This dilatory response is mediated via adrenergic beta receptors which can be blocked by propranolol (Paper 8).

The structural findings of a neuro-muscular complex within the wall of the Graafian follicle were confirmed by in vitro experiments showing that the smooth muscle system can be brought into function when the local

adrenergic nerves are stimulated to release their transmitter.

VI. PHARMACOLOGICAL CHARACTERIZATION OF ADRENERGIC AND CHOLINERGIC RECEPTORS IN THE OVARIAN SMOOTH MUSCULATURE, WITH SPECIAL REGARD TO THE FOLLICULAR WALL

Since the report by Guttmacher and Guttmacher (1921), ovarian contractile mechanisms have been studied under a variety of conditions. As a basis for further investigations on the functional significance of the intra-ovarian neuro-muscular complex, the smooth muscle receptors mediating the action of the autonomic innervation have been characterized in detail.

Definition and characterization of autonomic receptors

Our knowledge about the mechanisms for the interaction between drugs and receptors, as an operational approach to characterize various amine receptor mechanisms, is to a large extent based on the fundamental studies by Furchgott; several excellent reviews are available, the one from 1972 being particularly comprehensive. A drug-receptor system is composed of the tissue component that interacts with the agonist, and the complete sequence of events that connect the receptor with the response mechanism. The way to identify the presence of receptor sites is either to obtain responses to various agonists and compare the potencies or to show that the response can be antagonized by specific receptor blocking agents. As long as the true receptors have not yet been isolated, we have to depend on the assumption that similarities or differences in their response also reflect similarities or differences in the characteristics of the receptors mediating these responses. In this way, numerous examples of pharmacological receptor types have been discovered in smooth muscle tissue.

Schild (1947) has demonstrated that responses to agonists interacting with the same receptor are blocked to a similar degree by a given competitive antagonist. From this follows that the action of an agonist on various

tissues will be antagonized to the same extent by a competitive blocking agent if the receptors for the agonist in those tissues are identical. To quantify this relationship, Schild introduced the concept of pA_2 , i.e. the logarithm for the concentration of the antagonist necessary to increase the ED_{50} (dose producing half maximum response) by a factor of 2. A similar measure of antagonism is represented by the dissociation constant (K_B) of the receptor-antagonist complex, which is the relation between the concentration of antagonist and dose ratio minus 1. Another quantitative means to define the receptors is by the dissociation constant (K_A) for the receptor-agonist interaction. This requires the use of an irreversible inhibitor (e.g. dibenamine or phenoxybenzamine) which can block a fraction of the receptors. The doses producing equal responses before and after this blockade can be used to calculate K_A (Furchgott 1972). Thus, the determination of values for the dissociation constants provides a powerful method for both classifying agonists according to the receptors on which they act, and for classifying receptors on which a given agonist acts.

Contractility of ovarian tissue

It was reported by Rocereto et al. (1969) that the spontaneous rhythmic contractions of feline ovarian tissue examined in vitro could be increased by noradrenaline and adrenaline in low doses, while isoprenaline caused a reduction of the spontaneous contractions. Experiments with adrenergic blocking agents indicated that the stimulatory action induced by noradrenaline was mediated by adrenergic alpha receptors, and that adrenergic beta receptors were involved in the inhibitory effect of isoprenaline. However, as shown in Paper 1, the composite ovarian tissue provides a very complex model for studies of ovarian smooth muscle pharmacology, and marked species variation in the sympathomimetic response is encountered.

Isolated intact cat and guinea-pig ovaries were found to exhibit several patterns of spontaneous rhythmic contractility in vitro (Paper 1). Adrenaline and noradrenaline increased, whereas isoprenaline decreased, ovarian tension in the cat, confirming the observations on this animal by Rocereto et

al. (1969). In the guinea-pig ovaries, on the other hand, all three catecholamines reduced the tension. The spontaneous contractility present in both the cat and guinea-pig ovaries made it difficult to construct full dose-response curves. None the less, attempts were made to estimate the relative potency for various agonists on the basis of "dose for comparable response". This value was defined as the lowest dose producing at least half maximum response for agonists stimulating contraction, and the lowest dose abolishing contraction for those agonists which inhibit the spontaneous contractile activity. Using this method, the order of potency for the various drugs tested in respect of their relaxatory effect on guinea-pig ovaries was found to be isoprenaline > adrenaline > noradrenaline, indicating that adrenergic beta receptors mediate this type of response (Ahlquist 1948). When the beta receptors were blocked by INPEA, both adrenaline and noradrenaline caused increased contractile activity, adrenaline being the most potent of the two also in this respect. This indicates that adrenergic alpha receptors are involved in the contractile response (Ahlquist 1948), and it was also confirmed in experiments with the alpha-blocking-agent, phenoxybenzamine. Also for the cat ovary, the involvement of adrenergic alpha and beta receptors was demonstrated in the same way. In the guinea-pig, the beta receptor effect dominates over the alpha effect, whereas the situation is the opposite in the cat ovary.

In both species, acetylcholine increased ovarian tension by a mechanism that could be inhibited by atropine.

Contractility of follicle strip preparations

In order to isolate the pharmacological responses to that system of ovarian smooth muscle present in the follicular wall, strips from the protruding part of bovine and human Graafian follicles were dissected out and mounted in an organ bath for registration of motor activity as described in Section V. Usually no sign of spontaneous contraction was found in these isolated preparations; in a few cases only small rhythmic undulations could be observed (Paper 6). In order to allow for a quantitative characterization of the

receptors, neuronal uptake of the amines was blocked by cocaine, and nor-metanephrine was present in the bath to avoid any extraneuronal uptake.

Adrenergic alpha receptors. Both the bovine and human preparations always reacted with dose-dependent contraction when noradrenaline, adrenaline, phenylephrine or isoprenaline was injected into the organ bath. The order of potency for these amines (tested when the beta receptors were blocked) was found to be noradrenaline > adrenaline > phenylephrine > isoprenaline (for both species), indicating that the contractile response was mediated by adrenergic alpha receptors. On the basis of experiments with specific alpha-blocking agents the dissociation constants could be calculated both for the agonist (noradrenaline)-receptor complex (K_A) and for the antagonist (piperoxan)-receptor complex (K_B). These values were found to be $2.26 \times 10^{-6} M$ and $1.02 \times 10^{-8} M$, respectively, for the bovine follicular wall and $1.68 \times 10^{-6} M$ and $7.90 \times 10^{-8} M$, respectively, for the human follicular wall. These figures are in agreement with corresponding values given for other smooth muscle tissues, thus confirming that a system of conventional alpha-adrenergic receptors is present in the follicular smooth musculature.

Adrenergic beta receptors. Before testing the relaxing effect of the adrenergic agonists, the contractile adrenoceptors were irreversibly blocked by pretreatment of the preparation with dibenamine during 30-40 min. No relaxation of the strip was seen when adrenergic drugs were added to the bath after the preparation had accommodated to a steady level of tension. An active load was therefore induced by carbamylcholine, which produced an increased level of tension. In the human preparations the increased tension had a long enough duration to allow for recording of the relaxation induced by sympathomimetic compounds (Paper 6). The contraction induced in the bovine follicle strip did not, however, remain constant for the time needed to get full dose-response curves with the relaxatory agonists (Paper 3). Therefore, in the experiments with bovine preparations, carbamylcholine at a concentration of $5 \times 10^{-2} M$ was infused continuously into the organ bath at a rate of 15 $\mu l/min$. The strip then slowly reached a steady level of

tension which lasted long enough to test the relaxatory effect of sympathomimetics in their full dose ranges.

The agonists tested reduced the tension of the follicle strip in a dose-dependent manner. With the bovine preparation, the potency rank was found to be isoprenaline > adrenaline > noradrenaline > terbutaline. This order of potency differed from that found for the human follicle tissue, which was isoprenaline > noradrenaline > terbutaline > adrenaline. The competitive reversible beta antagonist, propranolol, shifted the relaxing terbutaline response in a parallel manner towards higher concentrations. On the basis of this shift the K_B values for the reaction with propranolol were determined and found to be 7.28×10^{-9} M and 5.42×10^{-7} M for the bovine and human preparations, respectively.

The results show that adrenergic beta receptors mediate the relaxatory response to sympathomimetics; they do not, however, provide any definite conclusion whether the receptors should be classified as the beta₁ or the beta₂ subtype. On the basis of comparison of relative potencies for noradrenaline and adrenaline, the bovine beta receptors should be of the beta₂ type while the human receptors should be classified as the beta₁ type (Furchgott 1972). On the other hand, the relative potencies found for isoprenaline and terbutaline would rather favour the presence of beta₁ receptors in the bovine and beta₂ receptors in the human preparation (Persson and Olsson 1970). The fact that the selective beta₁-receptor antagonist, practolol, does not influence the terbutaline response in the human follicle strip would agree with the presence of the beta₂ subtype of receptor in this preparation. The low K_B value found for the bovine preparation is rather close to the corresponding value for the complex between propranolol and the beta₁ receptor in the cat's pial artery (Edvinsson and Owman 1974), which supports the view that the beta receptors are more likely of the beta₁ subtype in the cow follicle.

Cholinergic receptors. Already Guttmacher and Guttmacher (1921) had found that physostigmine induces contraction of the porcine follicular wall.

Later studies by, e.g., Diaz-Infante et al. (1974), Okamura et al. (1974), O'Shea and Phillips (1974), and Walles et al. (1974 and Paper 1) have demonstrated that ovarian tissue (including follicular wall preparations) from a variety of mammalian species contracts when exposed to acetylcholine in vitro, and that this response to acetylcholine is inhibited by atropine. No attempts were, however, made in these studies to characterize the cholinergic receptors by means of quantitative methods.

Using the follicle strip preparation from bovine and human ovaries as described above, the cholinergic agonists, acetylcholine and carbamylcholine, were tested for their motor action on the smooth musculature (Papers 5 and 6). Both compounds always induced dose-dependent contraction of the preparations, acetylcholine being the most potent. A relaxation was never obtained, not even under conditions when the strip had previously been given an active tonic contraction by noradrenaline (instead, the compounds produced a further contraction on top of that induced by noradrenaline). The nicotinic-receptor antagonist, hexamethonium, did not influence the contractile response, whereas the specific muscarinic-receptor-blocking agent, atropine, caused a parallel shift of the dose-response curves for the parasympathomimetics towards higher concentration of the agonists. The dissociation constant for the receptor-atropine complex (K_B), calculated on the basis of blocking experiments with carbamylcholine as agonist, was found to be 1.17×10^{-10} M in the human and 7.75×10^{-10} M in the bovine preparation. Both the order of potency for the parasympathomimetics (acetylcholine > carbamylcholine) and the K_B values agree well with what is reported for muscarinic receptors in other smooth muscle tissues (e.g. Steinsland et al. 1972, Arunlakshana and Schild 1959, Thron and Waud 1968).

Neostigmine, which inhibits acetylcholinesterase activity, caused significant potentiation of the contractile response induced by acetylcholine (but not of that induced by carbamylcholine), which shows that the acetylcholine added to the test system is partly metabolized by the presence of cholinesterase in the preparation (Paper 5).

Adrenergic alpha receptors and cholinergic (muscarinic) receptors mediating contraction of the follicular wall have been identified and characterized. Relaxation of the follicular wall is mediated by adrenergic beta receptors, and quantitative experiments with various agonists and specific beta-receptor antagonists indicate that the human beta receptors are of the beta₂ subtype, whereas in the bovine follicle they rather seem to belong to the beta₁ subtype.

VII. POSSIBLE FUNCTIONAL ROLE OF THE FOLLICULAR SMOOTH MUSCULATURE AND ITS AUTONOMIC INNERVATION

The major events within the ovary which are essential for ovulation — follicular maturation, selection of the preovulatory follicles, and disruption of the follicular wall — are all dependent upon a precisely programmed secretion of pituitary gonadotropins. However, the local mechanisms involved in this process are still not very well understood. A number of local ovarian factors have frequently been discussed, and postulated factors include (a) increase in intrafollicular pressure; (b) proteolytic enzyme action in the follicular wall; (c) increased distensibility and plasticity of ovarian intercellular collagen; (d) vascular changes in the stigma; and (e) mechanical actions exerted by the oviduct. Studies during the last decade on the existence of autonomic nerves in a functional relationship with a well-developed system of smooth muscle cells in the follicular wall have introduced a new type of local mechanism that can be involved in the series of events eventually leading to the rupture of the follicular wall at ovulation.

It is possible that this neuro-muscular mechanism is somehow involved in an influence on intrafollicular pressure. In fact, von Euler and Hammarström (1937/38) were the first to extract an adrenaline-like substance from ovarian tissue, and in a series of experiments subsequently carried out on perfused rabbit ovaries they found that both the intrafollicular pressure and

the perfusion pressure were increased in the presence of small amounts of adrenaline or acetylcholine (von Euler and Hammarström 1937/38). In order to minimize interference from the vascular bed, we have measured intrafollicular pressure in isolated, human follicles incubated in a Krebs-Ringer solution (Paper 4). The intrafollicular pressure was recorded with a pressure transducer connected to a narrow cannula that was introduced into the follicular antrum. A dose-dependent increase in intrafollicular pressure, resembling the increase in tension of isolated follicular strips (Paper 4), was found after addition of noradrenaline into the bath in cumulative doses (Paper 4). This indicates that the neuro-muscular complex present within the follicular wall might have an influence on the intrafollicular pressure, and that this pressure is not simply secondary to changes in ovarian blood pressure. There is also direct evidence from in vivo experiments that interference with adrenergic receptors at the ovarian level or local sympathetic denervation changes the process of follicular maturation and the number of rupturing follicles (Virutamasen et al. 1971, Bahr et al. 1974, Stefansson et al. 1977).

Even if it can be assumed that the smooth muscle cells constitute a key system in a mechanical function of the follicular wall, it is not necessary that it is simply implicated in increasing follicular pressure to produce rupture of the follicular wall and expulsion of the ovum: it is usually assumed that the intrafollicular pressure does not change in association with ovulation (e.g. Rondell 1970). It should be noticed, though, that such propositions are hitherto based on experiments that have been performed without knowledge about the presently described adrenergic mechanisms, which may be instrumental in an increase in both follicular wall tension and intrafollicular pressure. Another possibility is that the smooth musculature provides one mechanical basis for the maintenance of the constant intrafollicular pressure claimed to exist during the period preceding follicular rupture (Blandau and Rumery 1963). Any of these processes, primarily controlled by the gonadotropins, may be influenced by the local sympathetic nerves.

These nerves might also act on the follicular smooth muscle system in an entirely different way related, for example, to those changes in the vascular bed required to produce the apical ischemia which seems to form part of the rupture mechanism (Blandau 1966). It may even be that the nerves do not primarily control a motor function, but rather have a trophic influence on such processes as the follicular development, selection of the preovulatory follicles, or their transformation after ovulation.

VIII. GENERAL SUMMARY

It is generally assumed that ovarian functions, including the complex series of events leading to expulsion of the ovum, are controlled and influenced entirely by humoral factors. Using modern neurohistochemical techniques it has been shown in studies performed during the last decade that the ovarian parenchyma including the follicles is extensively supplied with autonomic nerves which, hence, provide a basis also for neurogenic influences on ovarian functions. It is evident that some of these nerves are related to the ovarian vascular bed. However, a large proportion of the intraovarian nerves supply an extensive system of non-vascular smooth muscle cells which form an important constituent of the follicular theca externa. There is reason to believe that the system of autonomic nerves and smooth muscle may be involved in the process of ovulation. As a basis for further functional studies on this problem the neuro-muscular complex has been investigated in a series of studies utilizing histochemical, electron microscopic, and pharmacological techniques. The following main results were obtained:

(1) The theca externa of the follicular wall contains elongated cells with a mainly meridional distribution having the ultrastructural appearance of smooth muscle cells, i.e. the presence of numerous myofilaments, dense bodies, and micropinocytotic vesicles. The contractile nature of the cells in the theca externa has found proof in the immunohistochemical demonstration of actin and myosin in their cytoplasm.

(2) Both adrenergic and cholinergic nerves can be identified histochemically in the ovary, and in the follicular wall they run in among the smooth muscle cells.

(3) Both types of autonomic nerves form close innervation contacts with the smooth muscle cells in the theca externa, in which neuro-muscular distances, as narrow as 20 nm, are frequently observed with the electron microscope.

(4) Transmural electrical stimulation of the nerves in isolated follicle strips in vitro has shown that sufficient amounts of adrenergic transmitter are released locally to produce efficient contraction of the follicular wall.

(5) The isolated ovary exhibits spontaneous rhythmic contractions in a variety of patterns. This activity can be enhanced by alpha-adrenergic and cholinergic receptor stimulation and diminished by activation of beta-adrenergic receptors.

(6) The autonomic receptor mechanisms have been characterized in detail using isolated strips from the follicular wall. These preparations showed the same types of main receptor functions as in the whole ovary. Their identity and characteristics were established by pharmacological determination of values for the dissociation constants of receptor-agonist and -antagonist complexes.

(7) Intraluminal measurements of the pressure in intact follicles suggested that alterations in follicular wall tension are directly associated with changes in intrafollicular pressure, which may thus be influenced by the autonomic nervous system.

ACKNOWLEDGEMENTS

The present studies have been carried out at the Department of Histology, University of Lund, Sweden.

I wish to express my gratitude to Professor Bengt Falck, Head of the Department of Histology, for his continuous interest, help and support which have made this work possible. My deep thanks are due to Professor Christer Owman, my teacher, co-worker and friend. It has been a pleasure to work under his excellent guidance. I am very grateful to my co-worker, docent Nils-Otto Sjöberg. His continuous support and his friendship have been of great importance for my work. Also the staff at the Department of Histology is gratefully acknowledged for their skilful help and assistance.

I express my gratitude to all my friends at the Department of Zoology in Lund, where I received my basic biological education. Special thanks go to Professor Erik Dahl for his interest and positive support throughout my work, and to docent Patrick Meurling, who introduced me into the field of biological research and since then has supported and aided me in many ways.

Professor Robert F. Furchgott, Department of Pharmacology, State University of New York, is gratefully acknowledged for valuable criticism and stimulating discussion.

The studies underlying this thesis were supported by the Ford Foundation (Grants No. 68-383 and 680-0383 A), the World Health Organization (Project No. 74066), the Faculty of Medicine at the University of Lund, and the Royal Physiographic Society in Lund.

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